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Collective experiences of adventitious viruses of animal-derived raw materials and what can be done about them

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Abstract

Contamination of animal-derived raw materials with viruses, mycoplasmas, bacteria and fungi is common. These contaminants can interfere with the diagnosis of viral infection, and vaccines produced using infected cell cultures could lead to seroconversion or disease in the vaccinated animal. The purity, safety and efficacy of viral vaccines requires testing of the ingredients, cell substrates and final product. Methods for detection of viruses, especially bovine viral diarrhea virus, in nutrient serum, cell cultures, seed viruses and viral vaccines, and the frequency of their detection at the Center for Veterinary Biologics are discussed.

Abbreviations: BVDV: Bovine Viral Diarrhea Virus; FBS: Fetal Bovine Serum; FA: fluorescent antibody.

Introduction

Contamination of cell cultures used in the production of viral vaccines and in various diagnostic and serologic testing can be introduced in several ways. Primary and continuous cell lines may be contaminated if the original material is infected (tissues and eggs), if the dissociation material (usually trypsin) is virally contaminated, or if the nutrient serum contains virus. BVDV is probably the most common contaminant, as it is common in the bovine population.

Bovine viral diarrhea virus (BVDV) is currently classified as a member of the pestivirus genus of the Flaviviridae (Francki, 1991). Its two naturally occurring biotypes can be differentiated by the induction of cytopathic effects (CPE) in cell cultures (Lee, 1957; Gillespie, 1960). Isolates of the virus are diverse genetically and antigenically, although cross-reaction between strains by neutralization and other serologic methods is extensive (Gillespie, 1961; Fernelius, 1971; Castrucci, 1975; Horzinek, 1981). There is also significant serologic cross-reaction between BVDV and the other two members of the pestivirus group, hog cholera virus and border disease virus of sheep (Darbyshire, 1960; Osburn, 1973; Plant, 1973). The *in utero* infection of bovine fetuses can result in the contamination of serum processed from the fetuses. Contamination with BVDV of the fetal bovine serum (FBS) used in a test system can lead to false-positive pestivirus isolation by immunocytochemical or other techniques, or false-negative isolation of other viruses due to interference or masking. Contamination of test system FBS with antibody can lead to false-negative isolation, or false-positive bovine BVDV serology results. Persistent infection with non-cytopathic strains can occur.

Use of BVDV-contaminated FBS in cell cultures can lead to the infection of the cell cultures (Nuttall, 1977). Cell lines which have been reported to be infected with BVDV (Horzineck, 1981; Potts, 1989) include embryonic bovine lung (EBL), embryonic bovine kidney (EBK), Madin-Darby bovine kidney (MDBK), bovine turbinates (BoTur), embryonic bovine trachea (EBTR), porcine kidney lines PK-15,PK-2a and MVPK-1, rabbit kidney-13, Crandell feline kidney, feline lung (Fc2Lu), the Vero strain of African green monkey kidney and several mosquito cells. Infection of the cells with BVDV can alter the growth characteristics and viral yield of the cell lines,

as well as leading to false- positive BVDV diagnosis by viral isolation.

Materials and methods

The techniques used by the Center for Veterinary Biologics Laboratory (CVB-L) to test fetal serum are those stated in 9 Code of Federal Regulations (9CFR) as 'Requirements for ingredients of animal origin used for production of biologics', plus additional testing. The testing is done sequentially, with any positive samples eliminated at each step. Initially, a virus neutralization test (1:2 dilution) of one to two animal pools is made. Negative samples are examined for the presence of virus by use in growth medium (15% by volume) for three passages on susceptible cells (MDBK, BoTur, EBK) with direct or indirect fluorescent antibody (FA) staining performed on the cells at each passage. Negative cultures are frozen and thawed three times and inoculated onto susceptible virus-free cells (MDBK, BoTur, EBK) for two passages and examined by FA for virus. Pools of four negative samples are ultracentrifuged sufficiently to pellet BVDV. The pellet is resuspended and inoculated onto susceptible cells as above and two cell culture passages are made, with a final examination by FA for BVDV. Only negative samples are retained. Samples meeting the criteria of being negative for antibody to BVDV by neutralization tests, negative to BVDV by FA on all tests (direct growth and examination, freeze-thaw and ultracentrifugation) and having satisfactory growth potential (non-toxic, normal cell morphology observed, no cytopathic effect and a normal time to confluency) for the indicator cells (usually MDBK and EBK), are then checked for antibody and interference to twenty viral agents (bovine viral diarrhea, infectious bovine rhinotracheitis, Parainfluenza 3, bluetongue and other bovine viruses; porcine rotavirus, pseudorabies, porcine parvovirus, transmissable gastroenteritis, rabies, canine parainfluenza, canine distemper and other canine viruses and feline corona, feline calicivirus and other feline viruses) and pooled for specific uses. Pools are checked for purity from bacteria, fungi and mycoplasma. Additionally, these pools are either heat-treated for 30 min at 56 °C, or gamma-irradiated at 25–35 kiloGrays at –40 °C (House, 1990; Hansen, 1993).

Testing of master cell stocks follows the 9CFR 'Requirements for cell lines used for the production of biologics', which includes a minimum of two subcultures over a period of twenty-one days while observing for CPE. The final culture is tested for cytopathogenic and/or hemadsorbing agents, and by fluorescent antibody staining for specific extraneous agents, including BVDV. An aliquot of the final cell culture is frozen and thawed three times and the disrupted cells are inoculated onto appropriate cell lines, including bovine cell lines susceptible to BVDV. The cells are observed for 14 days with at least one subculture, followed by tests for cytopathogenic and/or hemadsorbing agents, and a FA test for specific extraneous agents.

Master seed viruses and selected viral vaccines are tested according to 9CFR under 'Detection of extraneous agents in master seed virus'. Briefly, the test is the same as that described above for cell lines after disruption.

In the past few years an additional series of tests has been used to screen out contaminated lots of FBS. Our current supplier has used a combination of 1) initial indirect (IFA) and immunoperoxidase staining, 2) amplification by flask and roller bottle culture, and detection with IFA and immunoperoxidase staining and 3) serum neutralization testing for both BVDV antibody and virus, plus other selected viruses (IBR, PI3 and Reo).

Results

The frequency of virus or antibody detection in 1 L lots containing sera from no more than two fetuses in fiscal years from 1982 through 1990 ranged from 20 to 87% positives (Levings, 1990). A review of testing records for the period 1990 to 1997 shows a rejection rate ranging from 31.98 to 68.47% (see Table 1) for serum samples going through a prescreening consisting of viral isolation, immunoperoxidase staining and neutralization testing. Since 1988 the Cytology section of the CVB-L and the Diagnostic Virology Section of the NVSL have rejected 23 of 394 pretested 4–5 L serum lots for BVD virus; one for bovine herpesvirus-4, two for reovirus by IFA and one for BLV by agar-gel immunodiffusion, as well as three due to toxicity or poor growth, for a final rejection rate of 7.6%. In addition, 18 lots showed some interference with serum neutralization tests (15 for rabies, one for canine parainfluenza and two for feline panleukopenia).

One positive master cell stock was detected in 1986 out of 16 lines tested. None of 69 master cells were found positive in fiscal years 1987 to 1990 (Levings,

Table 1. Fetal bovine serum samples rejected by year (1990–1997)

	Year							
	1990	1991	1992	1993	1994	1995	1996	1997
Antibody	79	102	24	170	305	263	217	160
BVD virus	182	399	248	418	131	134	53	108
Rejected/total samples	266/621	506/919	275/860	658/961	447/853	422/854	309/734	272/516
Per cent rejected	42.8	55	32	68.5	52.4	49.4	42.1	52.7

1990). From 1991 to 1997 none of 81 cell lines were found positive. Prior to fiscal year 1986 this laboratory found EBK, PK2a,PK-15 and CRFK master cell stocks positive for BVDV.

In the last ten years various cell lines have been developed at the CVB or received as possible test substrates. All are treated as potentially contaminated until tested according to the protocol for master cell stocks. Among those found to be virally contaminated were MVPK-1, RK-13 and CRFK with BVDV, and ESN and Neuro2a with reovirus. Among primary cell cultures lamb testes and bovine primary lymph node and kidney were BVDV contaminated. In addition, bovine kidney, Vero, CRFK, MA104A, swine tonsil, swine lung and swine alveolar macrophages were found to be contaminated with mycoplasmas (*Acholeplasma laidlawii* and *Mycoplasma orale*).

One master seed virus was tested and found to be BVDV contaminated between 1986 and 1990. Extraneous BVDV tests of final product for fiscal years 1981 to 1990 had positive rates of 0 to 3.6% of the number tested, with no positives detected after 1983. Testing rates during that period dropped from 60% of eligible serials to 11%. During the past seven years vaccine lots have been found contaminated with bluetongue virus (Ianconescu, 1996) and BVDV, introduced by contaminated serum, and master seed viruses have been found contaminated with reovirus, also introduced by serum. Primary tissue culture derived from chicken eggs has been contaminated with avian reovirus, poxvirus and avian adenovirus, and reticuloendotheliosis virus and tenosynovitis virus have been isolated from avian vaccines produced on avian cell culture.

Discussion

Viral contamination of raw animal materials (FBS, eggs, tissues and other) is a significant problem for researchers, diagnosticians, and vaccine manufacturers. The results both prior to 1990 and since 1990 are similar to that reported by others (Horzineck, 1981) who have observed that from 20 to 50% of FBS was virus positive. Depending on the year up to 60% of sampled lots were either virus and/or antibody positive for BVDV. At the CVB-L, all lots of serum which are negative for both BVDV virus and antibody by the testing methods indicated above are still irradiated before being used in either testing or cell production.

Many cell types appear to be susceptible to BVDV contamination (Lee, 1957), including those from non-human and human primates, (Erickson, 1991) although viral replication may not occur (Xue, 1996; Black, 1997). Currently all cell lines are tested for BVDV by the techniques outlined in the Code of Federal Regulations. Others have suggested that this approach is only a minimum requirement (Black, 1997), and that enhanced testing will reduce the possibility that cell lines may be contaminated at a very low level, which might not be picked up by the above testing. BVDV can be isolated if the virus can be captured by inoculation onto a suitable susceptible substrate, not masked by antibody in the nutrient serum, and amplified by replication so that the increase in viral numbers produces a detectable effect in cell culture. For most materials the detection phase indicators are CPE, cell death, hemadsorption, giant cell and syncytia formation and vacuolation, or an immunoassay such as FA or immunoperoxidase staining. BVDV causes problems because although it captures and amplifies well, the detection phase can be variable. There are two biotypes of BVDV. The 1st type (for example,

NADL and Singer strains) causes a strong CPE, which can completely destroy the cell culture, but does not persist or chronically infect cell cultures. Included in this biotype is strain Oregon C-24V which causes a foamy, incomplete CPE, with no persistent infection. The second type is the one that is of most concern to vaccine producers and diagnosticians. It causes little or no CPE, and may cause persistent or chronic infections in cell culture. Examples of this biotype of BVDV are NY-1, Tn-131, and 890 strains. Among the additional enhancement techniques which may be used to detect these, and other strains of BVDV, are the use of the 9CFR as a minimum standard, plus inclusion of an FA stain at each passage, rather than just at 21 days; the use of a non-cytopathic strain (e.g. NY-1) as a positive control, rather than a cytopathic strain, or both; the use of a one to two day old, subconfluent cell culture of susceptible cells; and the use of a 100% acetone fixation, with no water rinse to remove buffer crystals, since the BVDV FA stain is of lower avidity than many other FA stains (Black, 1997). A good FA scope, with clean lenses and a mercury or xenon light source, along with a good FA conjugate which has not been freeze-thawed or exposed to light for extended periods of time will also enhance the ability to detect this virus. Additional techniques which may enhance a laboratory's ability to detect BVDV are IFA staining using monoclonal antibodies against both CPE and non-CPE strains and polymerase chain reaction (PCR) primers to detect virus as well as distinguish between the two BVDV genotypes and subtypes Ia and Ib. PCR is very sensitive, but sampling problems may make this a test that may not be biologically relevant, especially if it picks up inactivated particles. The threshold (Lower, 1991) for detection (1000 to 10 000 per mL^3) may also be above that contained in many materials (especially when only 25–50 μL are sampled) as opposed to the 15 to 100 mL of serum often used for cell growth and viral amplification before testing. It is possible to detect a nonCPE strain in a modified live virus CPE vaccine strain, using rapid passes on bovine turbinates to preferentially increase the nonCPE strain and then IFA and PCR to detect contaminants of different genotypes. For those contaminants of the same genotype as the vaccine strain, plaque purification and subsequent biotype testing of numerous isolates would be required. Currently the CVB has the expertise to do this kind of testing in special cases, but not the staffing to do this routinely. PCR testing should detect both live BVDV and fragments of BVDV nucleic acid, as may FA staining. The gold standard for detection of BVDV still remains virus isolation.

The routine testing of trypsin by the 9CFR also is probably a minimum standard. It has been reported that, while only 1 of 8 lots of trypsin was found positive by the 9CFR, an enhanced test detected porcine parvovirus in 5 of the same 8 lots (Black, 1997). This increase in detection rate was due to increasing the subculturing from a minimum of one time over 14 days to 5 times over six weeks (although later tests showed that 2 subcultures over 21 days was optimal), using a pH 9.5 buffer to solubilize and dissolve the powdered trypsin and by using roller bottles instead of stationary flasks. Together, these changes were reported to increase the sensitivity of the test 10-fold. The FA test showed a typical nuclear fluorescence.

There have been many changes noted in the past seven years. Quality assurance programs and filtration have improved the quality of the nutrient products available, better irradiation techniques have improved the radiation process, and improvements in staining and amplification techniques have increased our ability to detect virus in raw materials, cell and viral stocks, and vaccines. The ability to use techniques like immunoperoxidase staining, PCR, and monoclonal antibodies to increase specificity, the ability to differentiate between genotypes, and the ability to differentiate contaminant BVDV from product BVDV will improve quality control.

Quality assurance programs at firms that process raw materials and produce nutrient serums and other materials have improved. Serum lots that were previously submitted for testing are now quality controlled at the point of origin and/or processing, so there are fewer lots of serum to be rejected. Also irradiation is much more rigorously controlled, so more lots are satisfactory for use in cell production, resulting in less reliance on heat inactivation or chemical treatments. Biologics firms still have the primary responsibility for monitoring the ingredients used in the production of vaccines. If the serum used is antibody and virus negative (and irradiated), the master cell and master seeds are negative, and aseptic techniques are used in production, then pure vaccines should be produced.

Filtration has greatly improved the quality of serum and seems effective in removing almost all mycoplasmas, and reducing or eliminating many viruses (Art To Science, 1989). Porcine parvovirus, however, shows a limited titer decrease after filtration. It is still necessary to test any lots of serum before they are used in production or testing.

What lies ahead? As harmonization efforts continue (Hodgson, 1993; Sanhueza, 1996), panels of experts will probably be formed, to determine the testing and inspection strategies necessary. They may recommend that a) the current tests be continued, with sensitivity, reproducibility, and comparability agreed upon; b) the tests continue to be improved; or c) the current tests be eliminated and a new set of standards agreed upon. All of this involves risk assessment, (Sutmoller, 1997) and will involve such issues as test validation (Vicari, 1991) and the use of irradiation even after negative testing results. In addition, new tests, such as use of transgenic mice for prion isolation, continue to be developed. There are good testing methods already in place to screen raw animal products for the presence of viruses and other contaminants, such as the use of ELISA (Bock, 1997), PCR (Kappeler, 1996; Laamanen, 1997) and monoclonal antibodies, in conjunction with the standards of virus isolation and serum neutralization testing. Our challenge is to continue to improve them.

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